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<b>(21) International Application Number:</b> PCT/US89/01464 <b>(22) International Filing Date:</b> 7 April 1989 (07.04.89)  <b>(30) Priority data:</b> 179,197 8 April 1988 (08.04.88) US  <b>(71) Applicant:</b> GENETICS INSTITUTE, INC. [US/US]; 87 CambridgePark Drive, Cambridge, MA 02140 (US).  <b>(72) Inventors:</b> WANG, Elizabeth, A. ; 136 Wolf Rock Road, Carlisle, MA 01741 (US). WOZNEY, John, M. ; 59 Old Bolton Road, Hudson, MA 01749 (US). ROSEN, Vicki ; 344 Marlborough Street, Apt. 4, Boston, MA 02116 (US).  <b>(74) Agent:</b> KAPINOS, Ellen, J.; Genetics Institute, Inc., 87 CambridgePark Drive, Cambridge, MA 02140 (US).		<b>(81) Designated States:</b> AT (European patent), AU, BE (Euro- pean patent), BF (OAPI patent), BG, BJ (OAPI patent), CF (OAPI patent), CG (OAPI patent), CH (European patent), CM (OAPI patent), DE (European patent), DK, FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), HU, IT (European patent), JP, KR, LU (European patent), ML (OAPI patent), MR (OAPI patent), NL (European patent), NO, SE (European pa- tent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent).  <b>Published</b> <i>With international search report.</i>

**(54) Title:** BONE AND CARTILAGE INDUCTIVE COMPOSITIONS**(57) Abstract**

Purified BMP-3 proteins and processes for producing them are disclosed. Compositions thereof may be used in the treat-  
ment of bone and/or cartilage defects and in wound healing and related tissue repair.

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## BONE AND CARTILAGE INDUCTIVE COMPOSITIONS

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The present invention relates to a novel family of purified proteins designated BMP-3 proteins and processes for obtaining them. Compositions thereof may be used to induce bone and/or cartilage formation and in wound healing and tissue repair.

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The invention provides proteins, capable of stimulating, promoting or otherwise inducing cartilage and/or bone formation, substantially free from other mammalian proteins. Human BMP-3 proteins of the invention are characterized by containing the amino acid sequence set forth in Table II from at least amino acid #377 through amino acid #472. These proteins are capable of inducing cartilage and or bone formation.

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Human BMP-3 proteins are produced by culturing a cell transformed with a DNA sequence substantially as shown in Table II and recovering from the culture medium a protein containing substantially the 96 amino acid sequence as shown in Table II from amino acid # 377 through amino acid # 472.

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Members of the BMP-3 protein family may be further characterized by the ability to demonstrate cartilage and/or bone formation activity in the rat bone formation assay described below. In preferred embodiments the proteins of the invention demonstrate activity in this rat bone formation assay at a concentration of .5 $\mu$ g - 100 $\mu$ g/gram of bone. In more preferred embodiments these proteins demonstrate activity in this assay at a concentration of 1 $\mu$ g - 50 $\mu$ g/gram of bone. More particularly, these proteins may be characterized by the ability of 1 $\mu$ g of the protein to score at least +2 in the rat bone formation assay.

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Another aspect of the invention provides pharmaceutical compositions containing a therapeutically effective amount of a BMP-3 protein in admixture with a pharmaceutically acceptable vehicle or carrier. The compositions may be used for bone

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and/or cartilage formation and may also be used for wound healing and tissue repair. Compositions of the invention may further include other therapeutically useful agents such as the BMP proteins BMP-1, BMP-2A, and BMP-2B disclosed in PCT publication WO88/00205. Other therapeutically useful agents include growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), and transforming growth factors (TGF- $\alpha$  and TGF- $\beta$ ). The compositions may also include an appropriate matrix, for instance, for supporting the compositions and providing a surface for bone and/or cartilage growth.

The compositions may be employed in methods for treating a number of bone defects and periodontal disease and various types of wounds. These methods, according to the invention, entail administering to a patient needing such bone and/or cartilage formation, wound healing, or tissue repair an effective amount of a novel BMP-3 protein of the present invention. These methods may also entail the administration of a BMP-3 protein of the invention in conjunction with at least one of the novel BMP proteins disclosed in PCT publication WO88/00205. In addition, these methods may also include administration of a BMP-3 with other growth factors.

Still a further aspect of the invention are DNA sequences coding for expression of a BMP-3 protein. Such sequences include the sequence of nucleotides in a 5' to 3' direction illustrated in Tables I A and I B and II or DNA sequences which hybridize under stringent conditions with the DNA sequences of Tables I A and I B and II and encode a protein having the ability to induce cartilage and/or bone formation. It is preferred that such proteins be further characterized by the ability to demonstrate cartilage and/or bone formation activity in the rat bone formation assay described below. In preferred embodiments the proteins of the invention demonstrate activity in this rat bone formation assay at a concentration of .5 $\mu$ g - 100 $\mu$ g/gram of bone. In more preferred embodiments

these proteins demonstrate activity in this assay at a concentration of  $1\mu\text{g} - 50\mu\text{g}/\text{gram}$  of bone. More particularly, these proteins may be characterized by the ability of  $1\mu\text{g}$  of the protein to score at least +2 in the rat bone formation assay. Finally, allelic or other variations of the sequences of Tables I A and I B and II, whether such nucleotide changes result in changes in the peptide sequence or not, are also included in the present invention.

Still a further aspect of the invention is a vector containing a DNA sequence as described above in operative association with an expression control sequence therefor. Such vector may be employed in a novel process for producing a BMP-3 protein of the invention in which a cell line transformed with a DNA sequence encoding expression of a BMP-3 protein in operative association with an expression control sequence therefor, is cultured in a suitable culture medium and a BMP-3 protein is isolated and purified therefrom. This claimed process may employ a number of known cells both prokaryotic and eukaryotic as host cells for expression of the polypeptide.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description and preferred embodiments thereof.

#### Detailed Description of the Invention

The purified BMP-3 proteins of the present invention are produced by culturing a host cell transformed with a DNA sequence comprising substantially as shown in Table II from nucleotide #321 to nucleotide #1736 or a portion thereof and recovered from the culture medium. The recovered BMP-3 proteins are characterized by the 96 amino acid sequence of a substantially homologous sequence as amino acid # 377 to amino acid # 472 as shown in Table II. These proteins may be further characterized by the ability to demonstrate cartilage and/or bone formation activity in the rat bone

formation assay described below. In preferred embodiments they demonstrate activity in this rat bone formation assay at a concentration of  $.5\mu\text{g} - 100\mu\text{g}/\text{gram}$  of bone. In more preferred embodiments these proteins demonstrate activity in  
5 this assay at a concentration of  $1\mu\text{g} - 50\mu\text{g}/\text{gram}$  of bone. More particularly, these proteins may be characterized by the ability of  $1\mu\text{g}$  of the protein to score at least +2 in the rat bone formation assay. Encompassed within the BMP-3 family of proteins of the invention are multiple variant forms including  
10 dimers and monomers both precursor and mature forms.

The BMP-3 proteins provided herein also include proteins encoded by the sequences similar to those of Tables I A and I B and II, but into which modifications are naturally provided (e.g. allelic variations in the nucleotide sequence which may  
15 result in amino acid changes in the polypeptide) or deliberately engineered. For example, synthetic polypeptides may wholly or partially duplicate continuous sequences of the amino acid residues of Tables I A and I B and II. These sequences, by virtue of sharing primary, secondary, or tertiary  
20 structural and conformational characteristics with BMP-3 proteins of Tables I A and I B and II may possess biological properties in common therewith. Thus, they may be employed as biologically active substitutes for naturally-occurring BMP-3 polypeptides in therapeutic processes.

25 Other specific mutations of the sequences of BMP-3 described herein involve modifications of the glycosylation sites. The absence of glycosylation or only partial glycosylation results from amino acid substitution or deletion at one or both of the asparagine-linked glycosylation  
30 recognition sites present in the sequences of the BMP-3 shown in Tables I A and I B and II. The asparagine-linked glycosylation recognition sites comprise tripeptide sequences which are specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either  
35 asparagine-X-threonine or asparagine-X-serine, where X is

usually any amino acid. A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) results in non-glycosylation at the modified tripeptide sequence.

The present invention also encompasses the novel DNA sequences, free of association with DNA sequences encoding other proteinaceous materials, and coding on expression for a BMP-3 protein. These DNA sequences include those depicted in Tables I A and I B and II in a 5' to 3' direction and those sequences which hybridize under stringent hybridization conditions [see, T. Maniatis et al, Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory (1982), pages 387 to 389] to the DNA sequences of Tables I A and I B and II and demonstrate cartilage and/or bone formation activity. An example of one such stringent hybridization condition is hybridization at 4X SSC at 65°C, followed by a washing in 0.1 X SSC at 65°C for an hour. Alternatively, an exemplary stringent hybridization condition is in 50% formamide, 4 X SCC at 42°C.

Similarly, DNA sequences which code for a BMP-3 polypeptides coded for by the sequences of Tables I A and I B and II, but which differ in codon sequence due to the degeneracies of the genetic code or allelic variations (naturally-occurring base changes in the species population which may or may not result in an amino acid change) also encode the novel growth factors described herein. Variations in the DNA sequences of Tables I A and I B and II which are caused by point mutations or by induced modifications (including insertion, deletion, and substitution) to enhance the activity, half-life or production of the polypeptides encoded thereby are also encompassed in the invention.

Another aspect of the present invention provides a novel method for producing BMP-3 proteins. The method of the present invention involves culturing a suitable cell or cell

line, which has been transformed with a DNA sequence coding on expression for a BMP-3 polypeptide of the invention, under the control of known regulatory sequences recovering and purifying the proteins from the culture medium. Suitable  
5 cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO). The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. See, e.g., Gething and  
10 Sambrook, Nature, 293:620-625 (1981), or alternatively, Kaufman et al, Mol. Cell. Biol., 5(7):1750-1759 (1985) or Howley et al, U.S. Patent 4,419,446. Another suitable mammalian cell line, which is described in the accompanying examples, is the monkey COS-1 cell line. The mammalian cell line CV-1  
15 may also be useful.

Bacterial cells may also be suitable hosts. For example, the various strains of E. coli (e.g., HB101, MC1061) are well-known as host cells in the field of biotechnology. Various strains of B. subtilis, Pseudomonas, other bacilli  
20 and the like may also be employed in this method.

Many strains of yeast cells known to those skilled in the art may also be available as host cells for expression of the polypeptides of the present invention. Additionally, where desired, insect cells may be utilized as host cells in the  
25 method of the present invention. See, e.g. Miller et al, Genetic Engineering, 8:277-298 (Plenum Press 1986) and references cited therein.

Another aspect of the present invention provides vectors for use in the method of expression of these novel BMP-3  
30 polypeptides. Preferably the vectors contain the full novel DNA sequences described above which code for the novel BMP-3 factors of the invention. Additionally the vectors also contain appropriate expression control sequences permitting expression of the BMP-3 protein sequences. Alternatively, vectors  
35 incorporating modified sequences as described above are also



embodiments of the present invention and useful in the production of the BMP-3 proteins. The vectors may be employed in the method of transforming cell lines and contain selected regulatory sequences in operative association with the DNA coding sequences of the invention which are capable of directing the replication and expression thereof in selected host cells. Useful regulatory sequences for such vectors are known to one of skill in the art and may be selected depending upon the selected host cells. Such selection is routine and does not form part of the present invention. Host cells transformed with such vectors and progeny thereof for use in producing BMP-3 proteins of the invention are also provided by the invention. Furthermore, proteins of the invention may be coexpressed with other "BMP" proteins such as those disclosed in WO88/00205.

A protein of the present invention, which induces cartilage and/or bone growth in circumstances where bone is not normally formed, has application in the healing of bone fractures and cartilage defects in humans and other animals. Such a preparation employing a BMP-3 protein may have prophylactic use in closed as well as open fracture reduction and also in the improved fixation of artificial joints. BMP-3 preparations of the invention may also be useful in the treatment of osteoporosis. De novo bone formation induced by an osteogenic agent contributes to the repair of congenital, trauma induced, or oncologic resection induced craniofacial defects, and also is useful in cosmetic plastic surgery. A BMP-3 protein of the invention may be valuable in the treatment of periodontal disease, and in other tooth repair processes. Such agents may provide an environment to attract bone-forming cells, stimulate growth of bone-forming cells or induce differentiation of progenitors of bone-forming cells. A variety of osteogenic, cartilage-inducing and bone inducing factors have been described. See, e.g. European patent applications 148,155 and 169,016 for discussions thereof.

The proteins of the invention may also be used in wound healing and tissue repair in humans and other animals. The types of wounds include, but are not limited to burns, incisions, and ulcers. (See, e.g., PCT Publication WO84/01106 for discussion of wound healing and related tissue repair). Of course, the proteins of the invention may have other therapeutic uses.

A further aspect of the invention is a therapeutic method and composition for repairing fractures and other conditions related to cartilage and/or bone defects or periodontal diseases. In addition, the invention comprises therapeutic methods and compositions for wound healing and tissue repair. Such compositions comprise a therapeutically effective amount of a BMP-3 protein in admixture with a pharmaceutically acceptable vehicle, carrier or matrix. It is expected that BMP-3 proteins may act in concert with or perhaps synergistically with other related proteins and growth factors. The invention encompasses therapeutic methods and compositions comprising a BMP-3 protein in combination with other related proteins or growth factors. Therapeutic methods and compositions of the invention may therefore comprise a therapeutic amount of a BMP-3 protein with a therapeutic amount of at least one of the other "BMP" proteins disclosed in PCT publication WO88/00205. Such combinations may comprise separate molecules of the "BMP" proteins or heteromolecules comprised of different "BMP" protein moieties. For example, a method and composition of the invention may comprise a disulfide-linked dimer comprising a BMP-3 protein and another "BMP" protein described above. Further, a BMP-3 protein of the invention may be combined with other agents beneficial to the treatment of the cartilage and/or bone defect, wound or tissue in question. These agents include various growth factors such as epidermal growth factor (EGF), platelet derived growth factor (PDGF), transforming growth factors (TGF- $\alpha$  and TGF- $\beta$ ), insulin-like growth factor (IGF) and

fibroblast growth factor (FGF). The preparation and formulation of such physiologically acceptable protein compositions, having due regard to pH, isotonicity, stability and the like, is within the skill of the art. The therapeutic compositions of the invention are also presently valuable for veterinary applications due to the lack of species specificity in BMP proteins. Particularly domestic animals and thoroughbred horses in addition to humans are desired patients for such treatment with BMP-3 proteins.

The therapeutic method includes administering the composition topically, systematically, or locally as an implant or device. When administered, the therapeutic composition for use in this invention is, of course, in a pyrogen-free, physiologically acceptable form. Further, the composition may desirably be encapsulated or injected in a viscous form for delivery to the site of bone, cartilage or tissue damage. Topical administration may be suitable for wound healing and related tissue repair. Preferably, for bone and/or cartilage formation, the bone growth inductive factor composition would include a matrix capable of delivering the bone inductive factor to the site of bone and/or cartilage damage, providing a surface and support structure for the developing bone and/or cartilage and optimally capable of being resorbed into the body. Such matrices may be formed of materials presently in use for other implanted medical applications.

The choice of matrix material is based on, for example, biocompatibility, biodegradability, mechanical properties, cosmetic appearance and interface properties. The particular application of the BMP-3 compositions will determine the appropriate formulation. Potential matrices for the compositions may be biodegradable and chemically defined such as calcium sulfate, tricalciumphosphate, hydroxyapatite, polylactic acid, and polyanhydrides. Other potential materials are biodegradable and biologically well defined, such as bone

or dermal collagen. Further matrices are comprised of pure proteins or extracellular matrix components. Other potential matrices are nonbiodegradable and chemically defined, such as sintered hydroxyapatite, bioglass, aluminates, or other ceramics. Matrices may be comprised of combinations of any of the above mentioned types of material, such as polylactic acid and hydroxyapatite or collagen and tricalciumphosphate. The bioceramics may also be altered in composition, such as in calcium-aluminate-phosphate and processing to alter for example, pore size, particle size, particle shape, and biodegradability.

The dosage regimen will be determined by the attending physician considering various factors which modify the action of the BMP-3 protein, e.g. amount of bone weight desired to be formed, the site of bone damage, the condition of the damaged bone, the size of a wound, type of damaged tissue, the patient's age, sex, and diet, the severity of any infection, time of administration and other clinical factors. The dosage may vary with the type of matrix used in the reconstitution and the types of BMP proteins in the composition. The addition of other known growth factors, such as IGF-I (insulin like growth factor I), to the final composition, may also effect the dosage.

Generally, the dosage regimen for cartilage and/or bone formation should be in the range of approximately  $10$  to  $10^6$  nanograms of protein per gram of bone weight desired. Progress can be monitored by periodic assessment of bone growth and/or repair, for example, using x-rays, histomorphometric determinations and tetracycline labeling.

The following examples illustrate practice of the present invention in recovering and characterizing a bovine BMP-3 protein and employing it to recover corresponding human BMP-3 proteins, and in expressing BMP-3 proteins via recombinant techniques.

## EXAMPLE I

Isolation of Bovine Bone Inductive Factor

Ground bovine bone powder (20-120 mesh, Helitrex) is prepared according to the procedures of M. R. Urist et al.,  
5 Proc. Natl Acad. Sci USA, 70:3511 (1973) with elimination of some extraction steps as identified below. Ten kgs of the ground powder is demineralized in successive changes of 0.6N HCl at 4°C over a 48 hour period with vigorous stirring. The resulting suspension is extracted for 16 hours at 4°C with 50  
10 liters of 2M CaCl<sub>2</sub> and 10mM ethylenediamine-tetraacetic acid [EDTA], and followed by extraction for 4 hours in 50 liters of 0.5M EDTA. The residue is washed three times with distilled water before its resuspension in 20 liters of 4M guanidine hydrochloride [GuCl], 20mM Tris (pH 7.4), 1mM N-ethylmaleimide,  
15 1mM iodoacetamide, 1mM phenylmethylsulfonyl fluoride as described in Clin. Orthop. Rel. Res., 171: 213 (1982). After 16 to 20 hours the supernatant is removed and replaced with another 10 liters of GuCl buffer. The residue is extracted for another 24 hours.

20 The crude GuCl extracts are combined, concentrated approximately 20 times on a Pellicon apparatus with a 10,000 molecular weight cut-off membrane, and then dialyzed in 50mM Tris, 0.1M NaCl, 6M urea (pH7.2), the starting buffer for the first column. After extensive dialysis the protein is loaded  
25 on a 4 liter DEAE cellulose column and the unbound fractions are collected.

The unbound fractions are concentrated and dialyzed against 50mM NaAc, 50mM NaCl (pH 4.6) in 6M urea. The unbound fractions are applied to a carboxymethyl cellulose column. Protein not  
30 bound to the column is removed by extensive washing with starting buffer, and the material containing protein having bone and/or cartilage formation activity as measured by the Rosen-modified Sampath-Reddi rat bone formation assay (described in Example III below) is desorbed from the column by 50mM  
35 NaAc, 0.25mM NaCl, 6M urea (pH 4.6). The protein from this

step elution is concentrated 20- to 40- fold, then diluted 5 times with 80mM KPO<sub>4</sub>, 6M urea (pH6.0). The pH of the solution is adjusted to 6.0 with 500mM K<sub>2</sub>HPO<sub>4</sub>. The sample is applied to an hydroxylapatite column (LKB) equilibrated in 80mM KPO<sub>4</sub>, 6M urea (pH6.0) and all unbound protein is removed by washing the column with the same buffer. Protein having bone and/or cartilage formation activity as measured by the rat bone formation assay is eluted with 100mM KPO<sub>4</sub> (pH7.4) and 6M urea.

The protein is concentrated approximately 10 times, and solid NaCl added to a final concentration of 0.15M. This material is applied to a heparin - Sepharose column equilibrated in 50mM KPO<sub>4</sub>, 150mM NaCl, 6M urea (pH7.4). After extensive washing of the column with starting buffer, a protein with bone and/or cartilage formation activity is eluted by 50mM KPO<sub>4</sub>, 700mM NaCl, 6M urea (pH7.4). This fraction is concentrated to a minimum volume, and 0.4ml aliquots are applied to Superose 6 and Superose 12 columns connected in series, equilibrated with 4M GuCl, 20mM Tris (pH7.2) and the columns developed at a flow rate of 0.25ml/min. The protein demonstrating bone and/or cartilage inductive activity has a relative migration on SDS-PAGE corresponding to an approximately 28,000 to 30,000 dalton protein.

The above fractions from the superose columns are pooled, dialyzed against 50mM NaAc, 6M urea (pH4.6), and applied to a Pharmacia MonoS HR column. The column is developed with a gradient to 1.0M NaCl, 50mM NaAc, 6M urea (pH4.6). Active fractions are pooled and brought to pH3.0 with 10% trifluoroacetic acid (TFA). The material is applied to a 0.46 x 25cm Vydac C4 column in 0.1% TFA and the column developed with a gradient to 90% acetonitrile, 0.1% TFA (31.5% acetonitrile, 0.1% TFA to 49.5% acetonitrile, 0.1% TFA in 60 minutes at 1ml per minute). Active bone and/or cartilage forming material is eluted at approximately 40-44% acetonitrile. Aliquots of the appropriate active fractions are iodinated by one of the following methods: P. J. McConahey et

al, Int. Arch. Allergy, 29:185-189 (1966); A. E. Bolton et al, Biochem J., 133:529 (1973); and D. F. Bowen-Pope, J. Biol. Chem., 237:5161 (1982). The iodinated proteins present in these fractions are analyzed by SDS gel electrophoresis and urea Triton X 100 isoelectric focusing. At this stage, the bone inductive factor is estimated to be approximately 10-50% pure.

## EXAMPLE II

### 10 Characterization of Bovine Bone Inductive Factor

#### A. Molecular Weight

Approximately 20ug protein from Example I is lyophilized and redissolved in 1X SDS sample buffer. After 15 minutes of heating at 37°C, the sample is applied to a 15% SDS polyacrylamide gel and then electrophoresed with cooling. The molecular weight is determined relative to prestained molecular weight standards (Bethesda Research Labs). Immediately after completion, the gel lane containing the bone and/or cartilage forming material is sliced into 0.3cm pieces. Each piece is mashed and 1.4ml of 0.1% SDS is added. The samples are shaken gently overnight at room temperature to elute the protein. Each gel slice is desalted to prevent interference in the biological assay. The supernatant from each sample is acidified to pH 3.0 with 10% TFA, filtered through a 0.45 micron membrane and loaded on a 0.46cm x 5cm C4 Vydac column developed with a gradient of 0.1% TFA to 0.1% TFA, 90% CH<sub>3</sub>CN. The appropriate bone and/or cartilage inductive protein - containing fractions are pooled and reconstituted with 20mg rat matrix and assayed. In this gel system, the majority of bone and/or cartilage formation fractions have the mobility of a protein having a molecular weight of approximately 28,000 - 30,000 daltons.

#### B. Isoelectric Focusing

35 The isoelectric point of the protein having bone and/or

cartilage formation activity is determined in a denaturing isoelectric focusing system. The Triton X100 urea gel system (Hoeffer Scientific) is modified as follows: 1) 40% of the ampholytes used are Servalyte 3/10; 60% are Servalyte 7-9; and 2) the catholyte used is 40mM NaOH. Approximately 20ug of protein from Example I is lyophilized, dissolved in sample buffer and applied to the isoelectrofocusing gel. The gel is run at 20 watts, 10°C for approximately 3 hours. At completion the lane containing bone and/or cartilage inductive factor is sliced into 0.5 cm slices. Each piece is mashed in 1.0ml 6M urea, 5mM Tris (pH 7.8) and the samples agitated at room temperature. The samples are acidified, filtered, desalted and assayed as described above. The major portion of activity as determined by the Rosen-modified Sampath-Reddi assay migrates in a manner consistent with a pI of about 8.8 - 9.2.

#### C. Subunit Characterization

The subunit composition of the isolated bovine bone protein is also determined. Pure bone inductive factor is isolated from a preparative 15% SDS gel as described above. A portion of the sample is then reduced with 5mM DTT in sample buffer and re-electrophoresed on a 15% SDS gel. The approximately 28-30kd protein yields two major bands at approximately 18 - 20kd and approximately 16 - 18kd, as well as a minor band at approximately 28 - 30kd. The broadness of the two bands indicates heterogeneity caused most probably by glycosylation, other post translational modification, proteolytic degradation or carbamylation.

#### 30 EXAMPLE III

##### Rosen Modified Sampath-Reddi Assay

A modified version of the rat bone formation assay described in Sampath and Reddi, Proc. Natl. Acad. Sci. U.S.A., 80:6591-6595 (1983) is used to evaluate bone and/or cartilage activity of the bovine protein obtained in Example I and the



BMP-1 proteins of the invention. This modified assay is herein called the Rosen-modified Sampath-Reddi assay. The ethanol precipitation step of the Sampath-Reddi procedure is replaced by dialyzing (if the composition is a solution) or  
5 diafiltering (if the composition is a suspension) the fraction to be assayed against water. The solution or suspension is then redissolved in 0.1 % TFA, and the resulting solution added to 20mg of rat matrix. A mock rat matrix sample not treated with the protein serves as a control. This material is frozen and  
10 lyophilized and the resulting powder enclosed in #5 gelatin capsules. The capsules are implanted subcutaneously in the abdominal thoracic area of 21 - 49 day old male Long Evans rats. The implants are removed after 7 - 14 days. Half of each implant is used for alkaline phosphatase analysis [See,  
15 A. H. Reddi et al., Proc. Natl Acad Sci., 69:1601 (1972)].

The other half of each implant is fixed and processed for histological analysis. lum glycolmethacrylate sections are stained with Von Kossa and acid fuschin to score the amount of induced bone and cartilage formation present in each  
20 implant. The terms +1 through +5 represent the area of each histological section of an implant occupied by new bone and/or cartilage cells and matrix. A score of +5 indicates that greater than 50% of the implant is new bone and/or cartilage produced as a direct result of protein in the implant. A score  
25 of +4, +3, +2 and +1 would indicate that greater than 40%, 30%, 20% and 10% respectively of the implant contains new cartilage and/or bone.

The rat matrix samples containing 200 ng of protein obtained in Example I result in bone and/or cartilage formation  
30 that filled more than 20% of the implant areas that was sectioned for histology. This protein therefore scores at least +2 in the Rosen-modified Sampath-Reddi assay. The dose response of the matrix samples indicates that the amount of bone and/or cartilage formed increases with the amount of  
35 protein in the sample. The control sample did not result in

any bone and/or cartilage formation. The purity of the protein assayed is approximately 10-15% pure.

The bone and/or cartilage formed is physically confined to the space occupied by the matrix. Samples are also analyzed  
5 by SDS gel electrophoresis and isoelectric focusing as described above, followed by autoradiography. Analysis reveals a correlation of activity with protein bands at 28 - 30kd and a pI of approximately 8.8 - 9.2. To estimate the purity of the protein in a particular fraction an extinction coefficient of  
10 1 OD/mg-cm is used as an estimate for protein and the protein is run on SDS PAGE followed by silver staining or radioiodination and autoradiography.

#### EXAMPLE IV

##### 15 Bovine BMP-3

The protein composition of Example IIA of molecular weight 28 - 30kd is reduced in situ and digested with trypsin. Eight tryptic fragments are isolated by standard procedures having the following amino acid sequences:

20 Fragment 1: A A F L G D I A L D E E D L G

Fragment 2: A F Q V Q Q A A D L

Fragment 3: N Y Q D M V V E G

Fragment 4: S T P A Q D V S R

Fragment 5: N Q E A L R

25 Fragment 6: L S E P D P S H T L E E

Fragment 7: F D A Y Y

Fragment 8: L K P S N ? A T I Q S I V E

A less highly purified preparation of protein from bovine bone is prepared according to a purification scheme  
30 similar to that described in Example I. The purification basically varies from that previously described by omission of the DE-52 column, the CM cellulose column and the mono S column, as well as a reversal, in the order of the hydroxylapatite and heparin sepharose columns. Briefly, the  
35 concentrated crude 4 M extract is brought to 85% final

concentration of ethanol at 4 degrees. The mixture is then centrifuged, and the precipitate redissolved in 50 mM Tris, 0.15 M NaCl, 6.0 M urea. This material is then fractionated on Heparin Sepharose as described. The Heparin bound material is fractionated on hydroxyapatite as described. The active fractions are pooled, concentrated, and fractionated on a high resolution gel filtration (TSK 30000 in 6 M guanidinium chloride, 50 mM Tris, pH 7.2). The active fractions are pooled, dialyzed against 0.1% TFA, and then fractionated on a C4 Vydac reverse phase column as described. A small amount of  $^{125}\text{I}$  labeled counterpart is mixed with the sample at this stage and the whole preparation is reduced and electrophoresed on an SDS polyacrylamide acrylamide gel [Laemmli, U.K., Nature, 277:680-685 (1970)]. The protein corresponding to the 16-18kd band is located using wet gel autoradiography and fixed with methanol-acetic acid-water using standard procedures, briefly rinsed with water, then neutralized with 0.1M ammonium bicarbonate. Following dicing the gel slice with a razor blade, the protein is digested from the gel matrix by adding 0.2  $\mu\text{g}$  of TPCK-treated trypsin (Worthington) and incubating the gel for 16 hours at 37°C. The resultant digest is then subjected to RPHPLC using a C4 Vydac RPHPLC column and 0.1% TFA-water 0.1% TFA water-acetonitrile gradient. The resultant peptide peaks were monitored by UV absorbance at 214 and 280 nm and subjected to direct amino terminal amino acid sequence analysis using an Applied Biosystems gas phase sequenator (Model 470A). Tryptic fragments are isolated having the following amino acid sequences:

Fragment 9: S L K P S N H A T I Q S ? V

Fragment 10: S F D A Y Y C S ? A

Fragment 11: V Y P N M T V E S C A

Fragment 12: V D F A D I ? W

Tryptic Fragments 7 and 8 are noted to be substantially the same as Fragments 10 and 9, respectively.

Probes consisting of pools of oligonucleotides (or unique oligonucleotides) are designed on the basis of the amino acid sequences of the tryptic Fragments 9 (Probe #3), 10 (Probe #2), and 11 (Probe #1), according to the method of R. Lathe, J. Mol. Biol., 183(1): 1-12 (1985), and synthesized on an automated DNA synthesizer; the probes are then radioactively labeled with polynucleotide Kinase and  $^{32}\text{P}$ -ATP.

Probe #1: A C N G T C A T [A/G] T T N G G [A/G] T A

10 Probe #2: C A [A/G] T A [A/G] T A N G C [A/G] T C [A/G] A A

Probe #3: T G [A/G/T] A T N G T N G C [A/G] T G [A/G] T T

The standard nucleotide symbols in the above-identified probes are as follows: A, adenosine; C, cytosine; G, guanine; T, thymine; and N, adenosine or cytosine or guanine or thymine.

Because the genetic code is degenerate (more than one codon can code for the same amino acid), the number of oligonucleotides in a probe pool is reduced based on the frequency of codon usage in eukaryotes, the relative stability of G:T base pairs, and the relative infrequency of the dinucleotide CpG in eukaryotic coding sequences [See Toole et al., Nature, 312:342-347 (1984)].

A recombinant bovine genomic library is constructed as follows: Bovine liver DNA is partially digested with the restriction endonuclease enzyme Sau 3A and sedimented through a sucrose gradient. Size fractionated DNA in the range of 15-30kb is then ligated to the bacteriophage Bam HI vector EMBL3 [Frischauf et al, J. Mol. Biol., 170:827-842 (1983)]. The library is plated at 8000 recombinants per plate. Duplicate nitrocellulose replicas of the plaques are made and amplified according to a modification of the procedure of Woo et al, Proc. Natl. Acad. Sci. USA, 75:3688-91 (1978). 400,000 recombinants are screened in duplicate with Probe #1 which has been labeled with  $^{32}\text{P}$ . The probes are hybridized in

3M tetramethylammonium chloride (TMAC), 0.1M sodium phosphate pH6.5, 1mM EDTA, 5X Denhardts, 0.6% SDS, 100ug/ml salmon sperm DNA at 48 degrees C, and washed in 3M TMAC, 50mM Tris pH8.0 at 50 degrees C. These conditions minimize the detection of mismatches to the 17 mer probe pool [see, Wood et al, Proc. Natl. Acad. Sci, U.S.A., 82:1585-1588 (1985)]. All recombinants which hybridized to this probe are replated for secondaries. Triplicate nitrocellulose replicas are made of the secondary plates, and amplified as described. The three sets of filters are hybridized to Probes #1, #2 and #3, again under TMAC conditions. One clone, lambda bP-819, hybridizes to all three probes and is plaque purified and DNA is isolated from a plate lysate. Bacteriophage lambda bP-819 was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockland, Maryland USA (hereinafter the ATCC) on June 16, 1987 under accession number 40344. This deposit meets the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and Regulations thereunder. This bP-819 clone encodes at least a portion of the bovine protein which we have designated BMP-3 or bBMP-3.

The region of bP-819 which hybridizes to Probe #2 is localized and sequenced. The partial DNA and derived amino acid sequences of this region are shown in Table IIA. The amino acid sequences corresponding to tryptic Fragments 10 and 12 are underlined. The first underlined sequence corresponds to Fragment 12 while the second corresponds to Fragment 10. This region of bP-819, therefore, which hybridizes to Probe #2 encodes at least 111 amino acids. This amino acid sequence is encoded by the DNA sequence from nucleotide #414 through #746.

TABLE I. A.

	383	393	403	413	(1)	428	
	GAGGAGGAAG	OGGTCTACGG	GGGTCTTCT	GCCTCTGCAG	AAC AAT GAG CTT CCT GGG GCA		
5					Asn Asn Glu Leu Pro Gly Ala		
	443		458		473		488
	GAA TAT CAG TAC AAG GAG GAT GAA GTA TGG GAG GAG AGG AAG CCT TAC AAG ACT						
10	Glu Tyr Gln Tyr Lys Glu Asp Glu Val Trp Glu Glu Arg Lys Pro Tyr Lys Thr						
		503		518		533	
	CTT CAG ACT CAG CCC CCT GAT AAG AGT AAG AAC AAA AAG AAA CAG AGG AAG GGA						
	Leu Gln Thr Gln Pro Pro Asp Lys Ser Lys Asn Lys Lys Lys Gln Arg Lys Gly						
	548		563		578		593
5	CCT CAG CAG AAG AGT CAG ACG CTC CAG TTT GAT GAA CAG ACC CTG AAG AAG GCA						
	Pro Gln Gln Lys Ser Gln Thr Leu Gln Phe Asp Glu Gln Thr Leu Lys Lys Ala						
		608		623		638	
10	AGA AGA AAG CAA TGG ATT GAA CCC CGG AAT TGT GCC AGA CGG TAC CTT AAA GTG						
	Arg Arg Lys Gln Trp Ile Glu Pro Arg Asn Cys Ala Arg Arg Tyr Leu Lys <u>Val</u>						
	653		668		683		698
	GAC TTC GCA GAT ATT GGC TGG AGC GAA TGG ATT ATT TCC CCC AAG TCC TTC GAT						
15	<u>Asp Phe Ala Asp Ile Gly Trp</u> Ser Glu Trp Ile Ile Ser Pro Lys <u>Ser Phe Asp</u>						
	713		728		743 (111)		756
	GCC TAT TAC TGC TCC GGA GCG TGC CAG TTC CCC ATG CCA AAG GTAGCCATTG						
	<u>Ala Tyr Tyr Cys Ser Gly Ala</u> Cys Gln Phe Pro MET Pro Lys						
20	766	776	786				
	TTTTTGTCC	TGTCCTTCCC	ATTTCATAG				

The region of bP-819 which hybridizes to Probe #1 and #3 is localized and sequenced. The partial DNA and derived amino acid sequences of this region are shown in Table IIB. The amino acid sequences corresponding to tryptic Fragments 9 and 11 are underlined. The first underlined sequence corresponds to Fragment 9 while the second underlined sequence corresponds to Fragment 11. The peptide sequence of this region of bP-819 which hybridizes to Probe #1 and #3 is 64 amino acids in length encoded by nucleotide #305 through #493 of Table IIB. The arginine residue encoded by the AGA triplet is presumed to be the carboxy-terminus of the protein based on the presence of a stop codon (TAA) adjacent to it. The nucleic acid sequence preceding the couplet TC (positions 305-306) is presumed to be an intron (non-coding sequence) based on the presence of a consensus acceptor sequence (i.e. a pyrimidine-rich stretch, TTCTCCCTTTTCGTTCT, followed by AG) and the presence of a stop rather than a basic residue in the appropriate position of the derived amino acid sequence.

bBMP-3 is therefore characterized by the DNA and amino acid sequence of Table I A and Table I B. The peptide sequence of this clone is 175 amino acids in length and is encoded by the DNA sequence from nucleotide #414 through nucleotide #746 of Table I A and nucleotide #305 through nucleotide #493 of Table I B.

TABLE I. B.

	284	294	304	(112)	319	
5	CTAACCTGTG	TTCTCCCTTT	TOGTTCCTAG	TCT TTG AAG CCA TCA AAT CAC GCT ACC		
				<u>Ser Leu Lys Pro Ser Asn His Ala Thr</u>		
	334	349	364	379		
	ATC CAG AGT ATA GTG AGA GCT GTG GGG GTC GTC CCT GGA ATC CCC GAG CCT TGC					
10	<u>Ile Gln Ser Ile Val</u>	Arg Ala Val Gly Val Val Pro Gly Ile Pro Glu Pro Cys				
	394	409	424	439		
	TGT GTG CCA GAA AAG ATG TCC TCA CTC AGC ATC TTA TTC TTT GAT GAA AAC AAG					
15	Cys Val Pro Glu Lys MET Ser Ser Leu Ser Ile Leu Phe Phe Asp Glu Asn Lys					
	454	469	484	(175)		
	AAT GTG GTA CTT AAA GTA TAT CCA AAC ATG ACA GTA GAG TCT TGT GCT TGC AGA					
	Asn Val Val Leu Lys <u>Val Tyr Pro Asn MET Thr Val Glu Ser Cys Ala Cys Arg</u>					
20	503	513	523	533		
	TAACCTGGTG	AAGAACTCAT	CTGGATGCTT	AACTCAATCG		



EXAMPLE VHuman BMP-3

The bovine and human BMP-3 genes are presumed to be significantly homologous, therefore a human genomic library is screened with two oligonucleotide probes synthesized with the bovine BMP-3 sequence above. The oligonucleotides are as follows

#1: d(AATTCGGGGTTCAATCCATTGCTTTCTTCTTGCCTTCTTCAGGGTCTCTGT)

#2: d(TTCGCTCCAGCCAATATCTGCGAAGTCCACTTTAAGGTACCGTCTGGCAC)

The oligonucleotides are synthesized on an automated synthesizer and radioactively labeled with polynucleotide kinase and <sup>32</sup>p-ATP. A human genomic library (Toole et al., supra) is plated. Duplicate nitrocellulose filter replicas of the library corresponding to 1,000,000 recombinants are made of and hybridized to the nick-translated probes in 5 X SSC, 5 X Denhardt's, 100ug/ml denatured salmon sperm DNA, 0.1% SDS (the standard hybridization solution) at 50 degrees centigrade for approximately 14 hours. The filters are then washed in 1 X SSC, 0.1% SDS at 50°C and subjected to autoradiography. Ten duplicate positives are isolated and plaque purified. Sequence analysis indicates that the positives contain the human BMP-3 gene.

A region comprised of the bovine DNA sequence residues 408-727 in Table I.A. is subcloned into the plasmid pSP65 [see D.A. Melton et al, Nucl. Acid Res., 12:7035-7056 (1984)], and amplified by standard techniques. The insert region of this plasmid is then excised and labeled with <sup>32</sup>p by nick-translation. A primer-extended cDNA library is made from the human lung small cell carcinoma cell line H128 (ATCC# HTB 120) using as a primer an oligonucleotide of the sequence d(AATGATTGAATTAAGCAATTC). This oligonucleotide was synthesized on the basis of the DNA sequence of the 3' untranslated region of the human BMP-3 gene. 375,000 recombinants from this library are screened with the nick-translated probe by standard methods. Recombinants from the library are hybridized

to the probe in standard hybridization solution at 65 and washed in 0.2 x SSc, 0.1% SDS at 65°C. 17 positives are obtained. One of these,  $\lambda$ H128-4 was deposited with the ATCC on March 31, 1988 under accession number 40437. This deposit meets the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and Regulations thereunder. The entire nucleotide sequence and derived amino acid sequence of the insert of H128-4 are given in Table II. This clone is expected to contain all of the nucleotide sequence necessary to encode the entire BMP-3 protein. The amino acid sequence of Table II is contemplated to represent a primary translation product which may be cleaved to produce the mature protein/s. Nucleotide #1 to #320 represents the 5' untranslated region and nucleotide #1736 to #1794 represents the 3' untranslated region. Precursor proteins may be cleaved at the proteolytic processing site between amino acid #360 and #361. The BMP-3 proteins encoded by Table II are contemplated to contain the 96 amino acid sequence from amino acid #377 to amino acid #472 or a sequence substantially homologous thereto. The sequences corresponding to tryptic Fragments 9-12 are underlined in Table II. The DNA sequence indicates that the human BMP-3 precursor protein is 472 amino acids. It is contemplated that BMP-3 corresponds to the approximately 16 to 18 kd subunit of Example IIC.

The sequences of BMP-3 as shown in Tables I A and I B and II, have significant homology to the beta (B) and beta (A) subunits of the inhibins. The inhibins are a family of hormones which are presently being investigated for use in contraception. See, A. J. Mason et al, Nature, 318:659-663 (1985). To a lesser extent they are also homologous to Mullerian inhibiting substance (MIS), a testicular glycoprotein that causes regression of the Mullerian duct during development of the male embryo and transforming growth factor-beta (TGF-  
b) which can inhibit or stimulate growth of cells or cause

them to differentiate. BMP-3 also demonstrates sequence similarity with Vgl. Vgl mRNA has been localized to the vegetal hemisphere of xenopus oocytes. During early development it is distributed throughout the endoderm, but the mRNA is  
5 not detectable after blastula formation has occurred. The Vgl protein may be the signal used by the endoderm cells to commit ectodermal cells to become the embryonic mesoderm. BMP-3 also shares some sequence similarity with the bone inductive protein BMP-2A disclosed in PCT publication  
10 WO88/00205.

TABLE II

	10	20	30	40	50	60	70
5	AGATCTTGAA	AACACCOGGG	CCACACACGC	CGCGACCTAC	AGCTCTTTCT	CAGCGTTTGA	GTGGAGAOCG
	80	90	100	110	120	130	140
10	CGCCCGCAGC	GCCCTGOGCG	GGTGAGGTCC	GCGCAGCTGC	TGGGGAAGAG	CCCACCTGTC	AGGCTGOGCT
	150	160	170	180	190	200	210
15	GGGTCAGOCG	AGCAAGTGGG	GCTGGCOGCT	ATCTOGCTGC	ACCGGGCOGC	GTCCCGGGCT	COGTGOGCCC
	220	230	240	250	260	270	280
20	TOGCCCCAGC	TGGTTTGGAG	TTCAACCCCTC	GGCTCCGCOG	COGGCTCCCTT	GCGCCTTGGG	AGTGTCCOGC
	290	300	310	320 (1)	335		
25	AGCGACGCOG	GGAGCOGACG	CGCOGOGOGG	GTACCTAGCC	ATG GCT GGG GCG AGC AGG CTG CTC		
					MET Ala Gly Ala Ser Arg Leu Leu		
	350	365	380	395			
30	TTT CTG TGG CTG GGC TGC TTC TGC GTG AGC CTG GCG CAG GGA GAG AGA CCG AAG CCA						
	Phe Leu Trp Leu Gly Cys Phe Cys Val Ser Leu Ala Gln Gly Glu Arg Pro Lys Pro						
	410	425	440	455			
35	CCT TTC CCG GAG CTC CGC AAA GCT GTG CCA GGT GAC CGC ACG GCA GGT GGT GGC COG						
	Pro Phe Pro Glu Leu Arg Lys Ala Val Pro Gly Asp Arg Thr Ala Gly Gly Gly Pro						
	470	485	500	515			
40	GAC TCC GAG CTG CAG CCG CAA GAC AAG GTC TCT GAA CAC ATG CTG OGG CTC TAT GAC						
	Asp Ser Glu Leu Gln Pro Gln Asp Lys Val Ser Glu His MET Leu Arg Leu Tyr Asp						
	530	545	560				
45	AGG TAC AGC ACG GTC CAG GCG GCC OGG ACA CCG GGC TCC CTG GAG GGA GGC TOG CAG						
	Arg Tyr Ser Thr Val Gln Ala Ala Arg Thr Pro Gly Ser Leu Glu Gly Gly Ser Gln						
	575	590	605	620			
50	CCC TGG CGC CCT CCG CTC CTG CGC GAA GGC AAC ACG GTT CGC AGC TTT OGG GCG GCA						
	Pro Trp Arg Pro Arg Leu Leu Arg Glu Gly Asn Thr Val Arg Ser Phe Arg Ala Ala						
	635	650	665	680			
55	GCA GCA GAA ACT CTT GAA AGA AAA GGA CTG TAT ATC TTC AAT CTG ACA TOG CTA ACC						
	Ala Ala Glu Thr Leu Glu Arg Lys Gly Leu Tyr Ile Phe Asn Leu Thr Ser Leu Thr						
	695	710	725	740			
60	AAG TCT GAA AAC ATT TTG TCT GCC ACA CTG TAT TTC TGT ATT GGA GAG CTA GGA AAC						
	Lys Ser Glu Asn Ile Leu Ser Ala Thr Leu Tyr Phe Cys Ile Gly Glu Leu Gly Asn						

755 770 785 800  
 ATC AGC CTG AGT TGT CCA GTG TCT GGA GGA TGC TCC CAT CAT GCT CAG AGG AAA CAC  
 Ile Ser Leu Ser Cys Pro Val Ser Gly Gly Cys Ser His His Ala Gln Arg Lys His  
 5  
 815 830 845  
 ATT CAG ATT GAT CTT TCT GCA TGG ACC CTC AAA TTC AGC AGA AAC CAA AGT CAA CTC  
 Ile Gln Ile Asp Leu Ser Ala Trp Thr Leu Lys Phe Ser Arg Asn Gln Ser Gln Leu  
 10 860 875 890 905  
 CTT GGC CAT CTG TCA GTG GAT ATG GCC AAA TCT CAT CGA GAT ATT ATG TCC TGG CTG  
 Leu Gly His Leu Ser Val Asp MET Ala Lys Ser His Arg Asp Ile MET Ser Trp Leu  
 920 935 950 965  
 15 TCT AAA GAT ATC ACT CAA TTC TTG AGG AAG GCC AAA GAA AAT GAA GAG TTC CTC ATA  
 Ser Lys Asp Ile Thr Gln Phe Leu Arg Lys Ala Lys Glu Asn Glu Glu Phe Leu Ile  
 980 995 1010 1025  
 20 GGA TTT AAC ATT ACG TCC AAG GGA CGC CAG CTG CCA AAG AGG AGG TTA CCT TTT CCA  
 Gly Phe Asn Ile Thr Ser Lys Gly Arg Gln Leu Pro Lys Arg Arg Leu Pro Phe Pro  
 1040 1055 1070 1085  
 GAG CCT TAT ATC TTG GTA TAT GCC AAT GAT GCC GCC ATT TCT GAG CCA GAA AGT GTG  
 Glu Pro Tyr Ile Leu Val Tyr Ala Asn Asp Ala Ala Ile Ser Glu Pro Glu Ser Val  
 25  
 1100 1115 1130  
 GTA TCA AGC TTA CAG GGA CAC OGG AAT TTT CCC ACT GGA ACT GTT CCC AAA TGG GAT  
 Val Ser Ser Leu Gln Gly His Arg Asn Phe Pro Thr Gly Thr Val Pro Lys Trp Asp  
 30 1145 1160 1175 1190  
 AGC CAC ATC AGA GCT GCC CTT TCC ATT GAG CGG AGG AAG AAG CGC TCT ACT GGG GTC  
 Ser His Ile Arg Ala Ala Leu Ser Ile Glu Arg Arg Lys Lys Arg Ser Thr Gly Val  
 1205 1220 1235 1250  
 35 TTG CTG CCT CTG CAG AAC AAC GAG CTT CCT GGG GCA GAA TAC CAG TAT AAA AAG GAT  
 Leu Leu Pro Leu Gln Asn Asn Glu Leu Pro Gly Ala Glu Tyr Gln Tyr Lys Lys Asp  
 1265 1280 1295 1310  
 40 GAG GTG TGG GAG GAG AGA AAG CCT TAC AAG ACC CTT CAG GCT CAG GCC CCT GAA AAG  
 Glu Val Trp Glu Glu Arg Lys Pro Tyr Lys Thr Leu Gln Ala Gln Ala Pro Glu Lys  
 1325 1340 1355 1370  
 AGT AAG AAT AAA AAG AAA CAG AGA AAG GGG CCT CAT OGG AAG AGC CAG ACG CTC CAA  
 Ser Lys Asn Lys Lys Lys Gln Arg Lys Gly Pro His Arg Lys Ser Gln Thr Leu Gln  
 45  
 1385 1400 1415  
 TTT GAT GAG CAG ACC CTG AAA AAG GCA AGG AGA AAG CAG TGG ATT GAA CCT OGG AAT  
 Phe Asp Glu Gln Thr Leu Lys Lys Ala Arg Arg Lys Gln Trp Ile Glu Pro Arg Asn  
 50 1430 1445 (377) 1460 1475  
 TGC GCC AGG AGA TAC CTC AAG GTA GAC TTT GCA GAT ATT GGC TGG AGT GAA TGG ATT  
 Cys Ala Arg Arg Tyr Leu Lys Val Asp Phe Ala Asp Ile Gly Trp Ser Glu Trp Ile

1490 1505 1520 1535  
ATC TCC CCC AAG TCC TTT GAT GCC TAT TAT TGC TCT GGA GCA TGC CAG TTC CCC ATG  
Ile Ser Pro Lys Ser Phe Asp Ala Tyr Tyr Cys Ser Gly Ala Cys Gln Phe Pro MET

5 1550 1565 1580 1595  
CCA AAG TCT TTG AAG CCA TCA AAT CAT GCT ACC ATC CAG AGT ATA GTG AGA GCT GTG  
Pro Lys Ser Leu Lys Pro Ser Asn His Ala Thr Ile Gln Ser Ile Val Arg Ala Val

10 1610 1625 1640 1655  
GGG GTC GTT CCT GGG ATT CCT GAG CCT TGC TGT GTA CCA GAA AAG ATG TCC TCA CTC  
Gly Val Val Pro Gly Ile Pro Glu Pro Cys Cys Val Pro Glu Lys MET Ser Ser Leu

15 1670 1685 1700  
AGT ATT TTA TTC TTT GAT GAA AAT AAG AAT GTA GTG CTT AAA GTA TAC CCT AAC ATG  
Ser Ile Leu Phe Phe Asp Glu Asn Lys Asn Val Val Leu Lys Val Tyr Pro Asn MET

1715 1730 (472) 1746 1756 1766 1776  
ACA GTA GAG TCT TGC GCT TGC AGA TAACCTGGCA AAGAACTCAT TTGAATGCCT AATTCAATCT  
20 Thr Val Glu Ser Cys Ala Cys Arg

1786  
CTAGAGTGGC CGGAATTC

EXAMPLE VIExpression of BMP-3

In order to produce bovine, human or other mammalian bone inductive factors, the DNA encoding it is transferred into an appropriate expression vector and introduced into mammalian cells or other preferred eukaryotic or prokaryotic hosts by conventional genetic engineering techniques. However the presently preferred expression system for biologically active recombinant human bone inductive factor is stably transformed mammalian cells.

One skilled in the art can construct mammalian expression vectors by employing the sequence of Tables I A and I B and II or other modified sequences and known vectors, such as pCD [Okayama et al., Mol. Cell Biol., 2:161-170 (1982)] and pJL3, pJL4 [Gough et al., EMBO J., 4:645-653 (1985)]. The transformation of these vectors into appropriate host cells can result in expression of a BMP-3 protein. One skilled in the art could manipulate the sequences of Tables I A and I B and II by eliminating or replacing the mammalian regulatory sequences flanking the coding sequence with bacterial sequences to create bacterial vectors for intracellular or extracellular expression by bacterial cells. For example, the coding sequences could be further manipulated (e.g. ligated to other known linkers or modified by deleting non-coding sequences therefrom or altering nucleotides therein by other known techniques). The modified BMP-3 coding sequence could then be inserted into a known bacterial vector using procedures such as described in T. Taniguchi et al., Proc. Natl Acad. Sci. USA, 77:5230-5233 (1980). This exemplary bacterial vector could then be transformed into bacterial host cells and BMP-3 expressed thereby. For a strategy for producing extracellular expression of BMP-3 in bacterial cells., see, e.g. European patent application EPA 177,343.

Similar manipulations can be performed for the construction of an insect vector [See, e.g. procedures described in published

European patent application 155,476] for expression in insect cells. A yeast vector could also be constructed employing yeast regulatory sequences for intracellular or extracellular expression of the factors of the present invention by yeast  
5 cells. [See, e.g., procedures described in published PCT application WO86/00639 and European patent application EPA 123,289].

A method for producing high levels of a BMP-3 protein factor of the invention from mammalian cells involves the  
10 construction of cells containing multiple copies of the heterologous BMP-3 gene. The heterologous gene can be linked to an amplifiable marker, e.g. the dihydrofolate reductase (DHFR) gene for which cells containing increased gene copies can be selected for propagation in increasing concentrations  
15 of methotrexate (MTX) according to the procedures of Kaufman and Sharp, J. Mol. Biol., 159:601-629 (1982). This approach can be employed with a number of different cell types. For example, a plasmid containing a DNA sequence for a BMP-3 protein of the invention in operative association with other  
20 plasmid sequences enabling expression thereof and the DHFR expression plasmid pAdA26SV(A)3 [Kaufman and Sharp, Mol. Cell. Biol., 2:1304 (1982)] can be co-introduced into DHFR-deficient CHO cells, DUKX-BII, by calcium phosphate coprecipitation and transfection, electroporation or protoplast  
25 fusion. DHFR expressing transformants are selected for growth in alpha media with dialyzed fetal calf serum, and subsequently selected for amplification by growth in increasing concentrations of MTX (sequential steps in 0.02, 0.2, 1.0 and 5uM MTX) as described in Kaufman et al., Mol Cell Biol.,  
30 5:1750 (1983). Transformants are cloned, and biologically active BMP-3 expression is monitored by rat bone formation assay described above in Example III. BMP-3 expression should increase with increasing levels of MTX resistance. Similar procedures can be followed to produce other BMP-3 family  
35 proteins.



### A. COS Cell Expression

As one specific example of producing a BMP-3 protein of Example V, the insert of H128-4 is released from the vector arms by digestion with KpnI, blunting with T4 polymerase, ligating on an EcoRI adapter, followed by digestion with Sal I. The insert is subcloned into the EcoRI and Xho I cloning sites of the mammalian expression vector, pMT2CXM. Plasmid DNA from this subclone is transfected into COS cells by the DEAE-dextran procedure [Sompayrac and Danna PNAS 78:7575-7578 (1981); Luthman and Magnusson, Nucl.Acids Res. 11: 1295-1308 (1983)] and the cells are cultured. Serum-free 24 hr. conditioned medium is collected from the cells starting 40-70 hr. post-transfection.

The mammalian expression vector pMT2 CXM is a derivative of p91023 (b) (Wong et al., Science 228:810-815, 1985) differing from the latter in that it contains the ampicillin resistance gene in place of the tetracycline resistance gene and further contains a XhoI site for insertion of cDNA clones. The functional elements of pMT2 CXM have been described (Kaufman, R.J., 1985, Proc. Natl. Acad. Sci. USA 82:689-693) and include the adenovirus VA genes, the SV40 origin of replication including the 72 bp enhancer, the adenovirus major late promoter including a 5' splice site and the majority of the adenovirus tripartite leader sequence present on adenovirus late mRNAs, a 3' splice acceptor site, a DHFR insert, the SV40 early polyadenylation site (SV40), and pBR322 sequences needed for propagation in E. coli.

Plasmid pMT2 CXM is obtained by EcoRI digestion of pMT2-VWF, which has been deposited with the American Type Culture Collection (ATCC), Rockville, MD (USA) under accession number ATCC 67122. EcoRI digestion excises the cDNA insert present in pMT2-VWF, yielding pMT2 in linear form which can be ligated and used to transform E. coli HB 101 or DH-5 to ampicillin resistance. Plasmid pMT2 DNA can be prepared by conventional

methods. pMT2CXM is then constructed using loopout/in mutagenesis (Morinaga, et al., Biotechnology 84: 636 (1984). This removes bases 1075 to 1145 relative to the starting from thr Hind III site near the SV40 origin of replication and enhancer sequences of pMT2. In addition it inserts the following sequence:

5' PO\_CATGGGCAGCTCGAG-3'

at nucleotide 1145. This sequence contains the recognition site for the restriction endonuclease Xho I, which is compatible with the Sal I site on the BMP-3 insert. Plasmid pMT2 CXM DNA may be prepared by conventional methods.

#### B. CHO Cell Expression

A BMP-3 protein of Example V may be expressed in CHO cells by releasing the insert of H128-4 from the vector arms by digestion with KpnI, blunting with T4 polymerase, ligating on an EcoRI adapter, followed by digestion with Sal I. The insert is subcloned into the EcoRI and Xho I cloning sites of the mammalian expression vector, pMT2CXM. Plasmid DNA from this subclone is transfected into CHO cells by electroporation [Neuman et al, EMBO J., 1:841-845 (1982)]. Two days later, cells are switched to selective medium containing 10% dialyzed fetal bovine serum and lacking nucleosides. Colonies expressing DHFR are counted 10-14 days later. Individual colonies or pools of colonies are expanded and analyzed for expression of BMP-3 RNA and protein using standard procedures and are subsequently selected for amplification by growth in increasing concentrations of MTX.

cdna genes inserted into the EcoRI and/or Xho I sites will be expressed as a bicistronic mRNA with DHFR in the second position. In this configuration, translation of the upstream (BMP-3) open reading frame is more efficient than the downstream (DHFR) cdna gene [Kaufman et al, EMBO J. 6:187-193 (1987). The amount of DHFR protein expressed is nevertheless sufficient for selection of stable CHO cell lines.

Characterization of the BMP-3 polypeptides through pulse labeling with [35S] methionine and polyacrylamide gel electrophoresis indicates that multiple molecular size forms of BMP-3 proteins are being expressed and secreted from the stable CHO lines.

#### Example VII

##### Biological Activity of Expressed BMP-3

To measure the biological activity of the expressed BMP-3 obtained in Example VI above, the BMP-3 is partially purified on a Heparin Sepharose column. 4 ml of the collected post transfection conditioned medium supernatant from one 100 mm dish is concentrated approximately 10 fold by ultrafiltration on a YM 10 membrane and then dialyzed against 20mM Tris, 0.15 M NaCl, pH 7.4 (starting buffer). This material is then applied to a 1.1 ml Heparin Sepharose column in starting buffer. Unbound proteins are removed by an 8 ml wash of starting buffer, and bound proteins, including BMP-3, are desorbed by a 3-4 ml wash of 20 mM Tris, 2.0 M NaCl, pH 7.4.

The proteins bound by the Heparin column are concentrated approximately 10-fold on a Centricon 10 and the salt reduced by diafiltration with 0.1% trifluoroacetic acid. The appropriate amount of this solution is mixed with 20 mg of rat matrix and then assayed for in vivo bone and/or cartilage formation activity by the Rosen-modified Sampath - Reddi assay. A mock transfection supernatant fractionation is used as a control for COS expressed proteins and for CHO expressed proteins CHO cell without BMP-3 conditioned medium fractionation is utilized. The implants containing rat matrix to which specific amounts of human BMP-3 have been added are removed from rats after seven days and processed for histological evaluation. Representative sections from each implant are stained for the presence of new bone mineral with von Kossa and acid fuschin, and for the presence of cartilage-specific matrix formation using toluidine blue.

The types of cells present within the section, as well as the extent to which these cells display phenotype are evaluated and scored as described in Example III.

5 Addition of human BMP-3 to the matrix material resulted in formation of cartilage-like nodules at 5 days post implantation. The chondroblast-type cells were recognizable by shape and expression of metachromatic matrix. The assay results indicate that BMP-3 proteins may be characterized by the ability of 1 $\mu$ g of the protein to score at least +2 in the  
10 rat bone formation assay. The amount of activity observed for human BMP-3 indicates that it may be dependent upon the amount of BMP-3 protein added to the matrix sample.

The procedures described above may be employed to isolate other related BMP-3 factors of interest by utilizing the  
15 bovine BMP-3 or human BMP-3 factors as a probe source. Such other BMP-3 proteins may find similar utility in, inter alia, fracture repair, wound healing and tissue repair.

The foregoing descriptions detail presently preferred embodiments of the present invention. Numerous modifications  
20 and variations in practice thereof are expected to occur to those skilled in the art upon consideration of these descriptions. Those modifications and variations are believed to be encompassed within the claims appended hereto.

What is claimed is:

1. A purified BMP-3 protein produced by the steps of
  - (a) culturing a cell transformed with a cDNA substantially  
5 as shown in Table II; and
  - (b) recovering from said culture medium a protein  
containing substantially the 96 amino acid sequence as  
shown in Table II from amino acid # 377 to amino acid #  
472.
- 10 2. A protein of claim 1 further characterized by the ability  
to induce cartilage and/or bone formation.
3. A protein of claim 1 further characterized by the ability  
15 of 1 $\mu$ g of said protein to score at least C +2 in the Rosen-  
modified Sampath-Reddi assay.
4. A cDNA sequence encoding a protein of claim 2.
- 20 5. A host cell transformed with a cDNA of claim 4.
6. A method for producing a purified BMP-3 protein said  
method comprising the steps of
  - (a) culturing in a suitable culture medium said transformed  
25 host cells of claim 5; and
  - (b) isolating and purifying said BMP-3 from said culture  
medium.
7. A pharmaceutical composition comprising an effective  
30 amount of a protein of claim 1 in admixture with a  
pharmaceutically acceptable vehicle.
8. A pharmaceutical formulation for bone and/or cartilage  
formation comprising an effective amount of a protein of  
35 claim 2 in a pharmaceutically acceptable vehicle.

9. A composition of claim 8 further comprising a matrix for supporting said composition and providing a surface for bone and/or cartilage growth.

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10. The composition of claim 9 wherein said matrix comprises a material selected from the group consisting of hydroxyapatite, collagen, polylactic acid and tricalcium phosphate.

10 11. A method for inducing bone and/or cartilage formation in a patient in need of same comprising administering to said patient an effective amount of the composition of claim 8.

12. A pharmaceutical composition for wound healing and tissue  
15 repair said composition comprising an effective amount of the protein of claim 1 in a pharmaceutically acceptable vehicle.

13. A method for treating wounds and/or tissue repair in a  
20 patient in need of same comprising administering to said patient an effective amount of the composition of claim 12.

14. An isolated DNA sequence encoding a BMP-3 protein said DNA sequence comprising substantially the nucleotide sequence or a portion thereof selected from the group consisting of:

- 25 (a) nucleotide #321 through nucleotide #1736  
(b) sequences which  
(1) hybridize to said sequence under stringent hybridization conditions; and  
(2) encode a protein characterized by the ability  
30 to induce cartilage and/or bone formation.

15. A DNA sequence of claim 14 further characterized by the ability of 1 $\mu$ g of said protein having the ability to score at least +2 in the Rosen-modified Sampath-Reddi assay.

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16. A vector comprising a DNA sequence of claim 14 in operative association with an expression control sequence therefor.

17. A host cell transformed with a DNA sequence of claim 14.

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18. A method for producing a BMP-3 protein, said method comprising the steps of

(a) culturing in a suitable culture medium said transformed host cell of claim 17; and

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(b) isolating and purifying said BMP-3 from said culture medium.

# INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US 89/01464**

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC <b>IPC(4): C12P 21/00; C07K 13/00; C07H 15/12</b> <b>US: 435/68; 530/350; 536/27</b>						
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; margin-top: 10px;">Minimum Documentation Searched <sup>7</sup></div> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 20%; padding: 5px;">Classification System</td> <td style="padding: 5px;">Classification Symbols</td> </tr> <tr> <td style="padding: 5px; vertical-align: top;"> <b>US</b> </td> <td style="padding: 5px;"> <b>435/68, 91, 172.1, 172.3, 320</b>   <b>536/27; 530/350; 935/18, 22, 33, 56</b> </td> </tr> </table> <div style="text-align: center; margin-top: 10px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup></div>			Classification System	Classification Symbols	<b>US</b>	<b>435/68, 91, 172.1, 172.3, 320</b>  <b>536/27; 530/350; 935/18, 22, 33, 56</b>
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<b>US</b>	<b>435/68, 91, 172.1, 172.3, 320</b>  <b>536/27; 530/350; 935/18, 22, 33, 56</b>					
<b>Chemical Abstracts Data Base (CAS) 1967-1989</b> <b>Key Words: bone morphogenic protein, pharmaceutical composition</b>						
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b>						
Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>				
Y	US, A, 4,619,989 (URIST) 28 October 1986, see abstract.	1-18				
P, Y	US, A, 4,795,804 (URIST) 03 January 1989, see abstract.	1-18				
Y, P	Proceedings of the National Academy of Sciences, U.S.A., Volume 85, issued December 1988 (WANG et al) "Purification and characterization of other distinct bone-inducing factors", see pages 9484-9488, see especially, the abstract.	1-18				
Y	WO, A WO86/00525 (SZABO) 30 January 1986 see abstract.	12-13				
Y	US, A, 4,394,370 (JEFFRIES) 19 July 1983, see abstract.	7-13				
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>						
<b>IV. CERTIFICATION</b>						
Date of the Actual Completion of the International Search  <b>09 June 1989</b>	Date of Mailing of this International Search Report  <div style="font-size: 1.5em; font-weight: bold;">17 JUL 1989</div>					
International Searching Authority  <b>ISA/US</b>	Signature of Authorized Officer  <div style="display: flex; align-items: center;"> <div style="flex: 1;">Joan Ellis</div> </div>					



## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	US,A, 4,563,350 (NATHAN et al)07 January 1986, see abstract.	7-13
Y,P	US,A, 4,789,732 (URIST)06 December 1988, see abstract.	7-13